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(57) Abstract

The invention discloses polynucleotides sequences coding for splice-variants of the human brain P2x4 receptor. The invention concerns also the recombinant expression of said receptors and their use for screening of antagonists.

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HUMAN P2x4 RECEPTOR SPLICE-VARIANTS

This invention relates to newly identified polynucleotides, polypeptides encoded by such polynucleotides, the use of such polynucleotides and polypeptides, as well as the production of such polynucleotides and polypeptides. More particularly, the polypeptide of the present invention is a novel human brain P_{2x} receptor and its splice variants. The invention also relates to modulating the action of such polypeptides and to the identification of drugs which modulate the actions of such polypeptides.

The P_2 purinoceptors have ATP as ligand. The P_{2x} subtype form a directly gated ion channel. Rat adult brain (Seguela, P. et. al., 1996, J. Neuroscience 16: 448-455), rat vas deferens (Valera, S. et. al., 1994, Nature 371: 516-519 and WO95/33048), human urinary bladder (WO95/33048), rat PC12 (Brake, A. J. et. al., 1994, Nature 371: 519-523) and rat sensory ganglion (Chen, C-C et al., 1995, Nature 377: 428-431, WO95/33048) forms of the P_{2x} receptor have been cloned.

In accordance with an aspect of the present invention, there is provided a polypeptide which is a human brain P_{2x} receptor polypeptide:

(a) having the deduced amino acid sequence of SEQ ID NO 3 or 4;

(b) characterised by the deduced amino acid sequence of SEQ ID NO 5;

(c) characterised by the amino acid sequence comprising residues 1 to 45 of SEQ ID NO 3; or

(d) or characterised by the amino acid sequence comprising residues 240-388 of SEQ ID NO 3;

or a fragment, analogue or derivative thereof.

Hereinafter the term polypeptide(s) will be used to refer to the human brain P_{2x} receptor and it fragments analogues and derivatives. The polypeptide having the deduced amino acid sequence of SEQ ID NO 3 is referred to herein as the P_{2x}-1 receptor, SEQ ID NO 4 as the the P_{2x}-2 receptor, and the human brain P_{2x} receptor polypeptide characterised by the deduced amino acid sequence of SEQ ID NO 5 as the P_{2x}-3 receptor.

In accordance with another aspect of the invention, there are provided polynucleotides (DNA or RNA) which encode such polypeptides.

In accordance with a preferred aspect of the invention, there is provided a polynucleotide which encodes a polypeptide of SEQ ID NO 3 or 4 or characterised by the deduced amino acid sequence of SEQ ID NO 5

In particular, the invention provides a polynucleotide having the DNA sequence given in SEQ ID NO 1 or 2.

mRNA encoding the P_{2X}-1 receptor was isolated from human brain frontal cortex mRNA. The predicted amino acid sequence of the P_{2X}-1 receptor has 388 amino acids and is 87, 51, 47 and 47% identical with rat adult brain (Seguela, P. et. al., 1996, J. Neuroscience 16: 448-455), rat vas deferens (Valera, S. et. al., 1994, Nature 371: 516-519), rat PC12 (Brake, A. J. et. al., 1994, Nature 371: 519-523) and rat sensory ganglion (Chen, C-C et al., 1995, Nature 377: 428-431) forms of the P_{2X} receptor. The pattern of hydrophobicity suggests two transmembrane regions and a large extracellular region for this human brain P_{2X} receptor. This agrees with that of the other P_{2X} receptors, and is consistent with the model of relatively short intracellular N- and C-termini. The position of the start codon (ATG) is in a Kozak consensus base sequence (Kozak, M., 1984, Nucleic Acid Research 12: 857-872) for translation initiation. Charged residues within the first 28 amino acids suggests the absence of a secretion leader.

mRNA encoding the P_{2X}-2 receptor was isolated from human brain frontal cortex. The P_{2X}-2 receptor is distinguished from P_{2X}-1 by the insertion of 48 base pairs between nucleotides 135 and 136 from the ATG start codon. The P_{2X}-2 mRNA encodes an inframe insertion of 16 amino acids between amino acid residues 45 and 46. The new insert sequence is [CYHLHLAEVEMESPRR]. This suggests the expression of a human brain P_{2X}-2 receptor isoform with a calculated molecular mass of 43.37 Kda. The N-terminus of this 16 amino acid insertion occurs towards the extracellular end of the first putative transmembrane region of the human P_{2X}-1 receptor. Sequence alignment, covering the end of the first transmembrane region and the junction of the extracellular domain, reveals a strong degree of sequence conservation between all P_{2X} receptors. Insertion of these additional amino acids, at this position, will have a significant effect on the conformation and number of ATP binding sites and hence, on the pharmacological and physiological properties of the human brain P_{2X} receptor.

EST Genbank database Accession Number R60722 was isolated from a 73 day postnatal female whole brain cDNA library. The encoded amino acid residues of EST (R60722), share a high degree of identity with the human brain P_{2X}-1 (residues 1 ~ 233). Residues 46 to 175 are deleted from the EST (R60722) as compared with the human brain P_{2X}-1 receptor. Further examination of this P_{2X} splice variant reveal the following points of interest:

1) The N-terminal position of the human brain P_{2X}-2 splice insertion and the EST (R60722) splice deletion coincides at exactly the same residue (YVIG↓WFV) within the first putative transmembrane domain. This highlights the fact that a variable region is located within the first transmembrane helix, extending into the extracellular portion of the

molecule. Splicing events in this region result in the generation of novel P_{2X} receptors and will have a profound effect on the pharmacology and physiology of the receptors.

2) The EST (R60722) deletion removes a substantial part of the extracellular region, including 6 of the 10 conserved cysteine residues involved in maintaining structural integrity of a putative ATP binding site.

This discovery of the sequence variability, (insertions or deletions) in the human brain P_{2X} receptor will be useful in designing highly specific novel receptor agonists or antagonists and for the development of novel diagnostics.

Sequence alignment of the predicted amino acid sequences of the human brain P_{2X} receptors of the invention with known receptors reveals several key conserved features:

1) A large extracellular region: The human brain P_{2X}-1 receptor contains a large extracellular region spanning ~ 270 amino acids. This region contains 10 conserved cysteines, present in all P_{2X} receptors, suggesting an important role in maintaining the three-dimensional structure of the receptor. The extracellular region contains 12 conserved glycines and 7 conserved lysine residues. These residues are commonly associated with nucleotide-binding sites (Traut, T. W., 1994, Eur. J. Biochem. 222, 9-19). Mutational data [Buell, G. et al EMBO, J. 15 55-62 (1996)] and the clustering of these residues supports the view that the extracellular region is involved in binding.

2) A Nucleotide-Binding Motif: The human brain P_{2X} sequences of this invention contain a sequence similar to the Walker type-A ATP-binding motif (Walker, J. et. al., 1982, EMBO J., 1, 945-951). These human brain P_{2X} receptors contain the ATP-binding motif [G(KA)GK(EDIIPTM)I] (the residues underlined are conserved in all known P_{2X} receptors).

3) An H5-like Pore Region: The human brain P_{2X} sequences of this invention contain an 8 amino acid sequence in the region preceeding and overlapping the second hydrophobic potential transmembrane domain. The human brain P_{2X} sequence, [TMINIGSG], closely resembles a motif which is conserved among all P_{2X} receptors. Further, this so-called H5 pore region is conserved in all voltage-gated potassium channels, inward rectifier potassium channels, and cyclic-nucleotide-gated cation channels (Ashford, M. L. et. al., 1994, Nature 370: 456-459).

The polynucleotide of the present invention may be in the form of RNA or in the form of DNA, which DNA includes cDNA, genomic DNA, and synthetic DNA. The DNA may be double-stranded or single-stranded, and if single stranded may be the coding strand or non-coding (anti-sense) strand. The coding sequence which encodes the mature polypeptide may be identical to the coding sequence shown in SEQ ID NO 1 or 2 or may be a different coding sequence which coding sequence, as a result of the

redundancy or degeneracy of the genetic code, encodes the same mature polypeptide as the DNA of SEQ ID NO 1 or 2.

The polynucleotide which encodes for the mature polypeptide of the invention may include: only the coding sequence for the mature polypeptide; the coding sequence for the mature polypeptide and additional coding sequence such as a leader or secretory sequence or a proprotein sequence; the coding sequence for the mature polypeptide (and optionally additional coding sequence) and non-coding sequence, such as introns or non-coding sequence 5' and/or 3' of the coding sequence for the mature polypeptide.

Thus, the term "polynucleotide encoding a polypeptide" encompasses a polynucleotide which includes only coding sequence for the polypeptide as well as a polynucleotide which includes additional coding and/or non-coding sequence.

The present invention further relates to variants of the hereinabove described polynucleotides which encode for fragments, analogs and derivatives of the mature polypeptide. The variant of the polynucleotide may be a naturally occurring allelic variant of the polynucleotide or a non-naturally occurring variant of the polynucleotide.

Thus, the present invention includes polynucleotides encoding the same mature polypeptide as well as variants of such polynucleotides which variants encode for a fragment, derivative or analog of the mature polypeptide. Such nucleotide variants include deletion variants, substitution variants and addition or insertion variants.

As hereinabove indicated, the polynucleotide may have a coding sequence which is a naturally occurring allelic variant of the coding sequence shown in SEQ ID NO 1 or 2. As known in the art, an allelic variant is an alternate form of a polynucleotide sequence which may have a substitution, deletion or addition of one or more nucleotides, which does not substantially alter the function of the encoded polypeptide.

The present invention also includes polynucleotides, wherein the coding sequence for the mature polypeptide may be fused in the same reading frame to a polynucleotide sequence which aids in expression and secretion of a polypeptide from a host cell, for example, a leader sequence which functions as a secretory sequence for controlling transport of a polypeptide from the cell. The polypeptide having a leader sequence is a preprotein and may have the leader sequence cleaved by the host cell to form the mature form of the polypeptide. The polynucleotides may also encode for a proprotein which is the mature protein plus additional 5' amino acid residues. A mature protein having a prosequence is a proprotein and is an inactive form of the protein. Once the prosequence is cleaved an active mature protein remains.

Thus, for example, the polynucleotide of the present invention may encode for a mature protein, or for a protein having a prosequence or for a protein having both a prosequence and a presequence (leader sequence).

The polynucleotides of the present invention may also have the coding sequence fused in frame to a marker sequence which allows for purification of the polypeptide of the present invention. The marker sequence may be a hexa-histidine tag supplied by a pQE-9 vector to provide for purification of the mature polypeptide fused to the marker in the case of a bacterial host, or, for example, the marker sequence may be a hemagglutinin (HA) tag when a mammalian host, e.g. COS-7 cells, is used. The HA tag corresponds to an epitope derived from the influenza hemagglutinin protein (Wilson, I., et al., Cell, 37:767 (1984)).

The present invention further relates to polynucleotides which hybridize to the hereinabove-described sequences under stringent conditions to the hereinabove-described polynucleotides. As herein used, the term "stringent conditions" means hybridization will occur only if there is at least 95% and preferably at least 97% identity between the sequences. The polynucleotides which hybridize to the hereinabove described polynucleotides in a preferred embodiment encode polypeptides which retain substantially the same biological function or activity as the mature polypeptide of the invention.

The polypeptide of the present invention may be a recombinant polypeptide, a natural polypeptide or a synthetic polypeptide, preferably a recombinant polypeptide.

The terms "fragment," "derivative" and "analogue" when referring to the mature polypeptide of the invention, means a polypeptide which retains essentially the same biological function or activity as such polypeptide. Thus, an analogue includes a proprotein which can be activated by cleavage of the proprotein portion to produce an active mature polypeptide.

The fragment, derivative or analogue of the mature polypeptide of the invention may be (i) one in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue (preferably a conserved amino acid residue) and such substituted amino acid residue may or may not be one encoded by the genetic code, or (ii) one in which one or more of the amino acid residues includes a substituent group, or (iii) one in which the mature polypeptide is fused with another compound, such as a compound to increase the half-life of the polypeptide (for example, polyethylene glycol), or (iv) one in which the additional amino acids are fused to the mature polypeptide, such as a leader or secretory sequence or a sequence which is employed for purification of the mature polypeptide or a proprotein sequence. Such

fragments, derivatives and analogs are deemed to be within the scope of those skilled in the art from the teachings herein.

The polypeptides and polynucleotides of the present invention are preferably provided in an isolated form, and preferably are purified to homogeneity.

The term "isolated" means that the material is removed from its original environment (e.g., the natural environment if it is naturally occurring). For example, a naturally-occurring polynucleotide or polypeptide present in a living animal is not isolated, but the same polynucleotide or polypeptide, separated from some or all of the coexisting materials in the natural system, is isolated. Such polynucleotides could be part of a vector and/or such polynucleotides or polypeptides could be part of a composition, and still be isolated in that such vector or composition is not part of its natural environment.

The present invention also relates to vectors which include polynucleotides of the present invention, host cells which are genetically engineered with vectors of the invention and the production of polypeptides of the invention by recombinant techniques.

In accordance with yet a further aspect of the present invention, there is therefore provided a process for producing the polypeptide of the invention by recombinant techniques by expresssing a polynucleotide encoding said polypeptide in a host and recovering the expressed product. Alternatively, the polypeptides of the invention can be synthetically produced by conventional peptide synthesizers.

Host cells are genetically engineered (transduced or transformed or transfected) with the vectors of this invention which may be, for example, a cloning vector or an expression vector. The vector may be, for example, in the form of a plasmid, a viral particle, a phage, etc. The engineered host cells can be cultured in conventional nutrient media modified as appropriate for activating promoters, selecting transformants or amplifying the human P2x receptor genes. The culture conditions, such as temperature, pH and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

The polynucleotides of the present invention may be employed for producing polypeptides by recombinant techniques. Thus, for example, the polynucleotide may be included in any one of a variety of expression vectors for expressing a polypeptide. Such vectors include chromosomal, nonchromosomal and synthetic DNA sequences, e.g., derivatives of SV40; bacterial plasmids; phage DNA; baculovirus; yeast plasmids; vectors derived from combinations of plasmids and phage DNA, viral DNA such as vaccinia, adenovirus, fowl pox virus, and pseudorabies. However, any other vector may be used as long as it is replicable and viable in the host.

The appropriate DNA sequence may be inserted into the vector by a variety of procedures. In general, the DNA sequence is inserted into an appropriate restriction endonuclease site(s) by procedures known in the art. Such procedures and others are deemed to be within the scope of those skilled in the art.

The DNA sequence in the expression vector is operatively linked to an appropriate expression control sequence(s) (promoter) to direct mRNA synthesis. As representative examples of such promoters, there may be mentioned: LTR or SV40 promoter, the E. coli. lac or trp, the phage lambda P_L promoter and other promoters known to control expression of genes in prokaryotic or eukaryotic cells or their viruses. The expression vector also contains a ribosome binding site for translation initiation and a transcription terminator. The vector may also include appropriate sequences for amplifying expression.

In addition, the expression vectors preferably contain one or more selectable marker genes to provide a phenotypic trait for selection of transformed host cells such as dihydrofolate reductase or neomycin resistance for eukaryotic cell culture, or such as tetracycline or ampicillin resistance in E. coli.

The vector containing the appropriate DNA sequence as hereinabove described, as well as an appropriate promoter or control sequence, may be employed to transform an appropriate host to permit the host to express the protein.

As representative examples of appropriate hosts, there may be mentioned: bacterial cells, such as E. coli, Streptomyces, Salmonella typhimurium; fungal cells, such as yeast; insect cells such as Drosophila and Sf9; animal cells such as CHO, COS or Bowes melanoma; plant cells, etc. The selection of an appropriate host is deemed to be within the scope of those skilled in the art from the teachings herein.

More particularly, the present invention also includes recombinant constructs comprising one or more of the sequences as broadly described above. The constructs comprise a vector, such as a plasmid or viral vector, into which a sequence of the invention has been inserted, in a forward or reverse orientation. In a preferred aspect of this embodiment, the construct further comprises regulatory sequences, including, for example, a promoter, operably linked to the sequence. Large numbers of suitable vectors and promoters are known to those of skill in the art, and are commercially available. The following vectors are provided by way of example. Bacterial: pQE70, pQE60, pQE-9 (Qiagen), pbs, pD10, phagescript, psiX174, pbluescript SK, pbsks, pNH8A, pNH16a, pNH18A, pNH46A (Stratagene); ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5 (Pharmacia). Eukaryotic: pWLNEO, pSV2CAT, pOG44, pXT1, pSG (Stratagene)

pSVK3, pBPV, pMSG, pSVL (Pharmacia). However, any other plasmid or vector may be used as long as they are replicable and viable in the host.

Promoter regions can be selected from any desired gene using CAT (chloramphenicol transferase) vectors or other vectors with selectable markers. Two appropriate vectors are PKK232-8 and PCM7. Particular named bacterial promoters include lacI, lacZ, T3, T7, gpt, lambda P_R, P_L and trp. Eukaryotic promoters include CMV immediate early, HSV thymidine kinase, early and late SV40, LTRs from retrovirus, and mouse metallothionein-I. Selection of the appropriate vector and promoter is well within the level of ordinary skill in the art.

In a further embodiment, the present invention relates to host cells containing the above-described constructs. The host cell can be a higher eukaryotic cell, such as a mammalian cell, or a lower eukaryotic cell, such as a yeast cell, or the host cell can be a prokaryotic cell, such as a bacterial cell. Introduction of the construct into the host cell can be effected by calcium phosphate transfection, DEAE-Dextran mediated transfection, or electroporation. (Davis, L., Dibner, M., Battey, I., Basic Methods in Molecular Biology, (1986)).

The constructs in host cells can be used in a conventional manner to produce the gene product encoded by the recombinant sequence. Alternatively, the polypeptides of the invention can be synthetically produced by conventional peptide synthesizers.

Mature proteins can be expressed in mammalian cells, yeast, bacteria, or other cells under the control of appropriate promoters. Cell-free translation systems can also be employed to produce such proteins using RNAs derived from the DNA constructs of the present invention. Appropriate cloning and expression vectors for use with prokaryotic and eukaryotic hosts are described by Sambrook, et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor, N.Y., (1989), the disclosure of which is hereby incorporated by reference.

Transcription of the DNA encoding the polypeptides of the present invention by higher eukaryotes is increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp that act on a promoter to increase its transcription. Examples including the SV40 enhancer on the late side of the replication origin bp 100 to 270, a cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

Generally, recombinant expression vectors will include origins of replication and selectable markers permitting transformation of the host cell, e.g., the ampicillin resistance gene of E. coli and S. cerevisiae TRP1 gene, and a promoter derived from a

highly-expressed gene to direct transcription of a downstream structural sequence. Such promoters can be derived from operons encoding glycolytic enzymes such as 3-phosphoglycerate kinase (PGK), _-factor, acid phosphatase, or heat shock proteins, among others. The heterologous structural sequence is assembled in appropriate phase with translation initiation and termination sequences, and preferably, a leader sequence capable of directing secretion of translated protein into the periplasmic space or extracellular medium. Optionally, the heterologous sequence can encode a fusion protein including an N-terminal identification peptide imparting desired characteristics, e.g., stabilization or simplified purification of expressed recombinant product.

Useful expression vectors for bacterial use are constructed by inserting a structural DNA sequence encoding a desired protein together with suitable translation initiation and termination signals in operable reading phase with a functional promoter. The vector will comprise one or more phenotypic selectable markers and an origin of replication to ensure maintenance of the vector and to, if desirable, provide amplification within the host. Suitable prokaryotic hosts for transformation include E. coli, Bacillus subtilis, Salmonella typhimurium and various species within the genera Pseudomonas, Streptomyces, and Staphylococcus, although others may also be employed as a matter of choice.

As a representative but nonlimiting example, useful expression vectors for bacterial use can comprise a selectable marker and bacterial origin of replication derived from commercially available plasmids comprising genetic elements of the well known cloning vector pBR322 (ATCC 37017). Such commercial vectors include, for example, pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden) and GEM1 (Promega Biotec, Madison, WI, USA). These pBR322 "backbone" sections are combined with an appropriate promoter and the structural sequence to be expressed.

Following transformation of a suitable host strain and growth of the host strain to an appropriate cell density, the selected promoter is induced by appropriate means (e.g., temperature shift or chemical induction) and cells are cultured for an additional period.

Cells are typically harvested by centrifugation, disrupted by physical or chemical means, and the resulting crude extract retained for further purification.

Microbial cells employed in expression of proteins can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents, such methods are well know to those skilled in the art.

Various mammalian cell culture systems can also be employed to express recombinant protein. Examples of mammalian expression systems include the COS-7 lines of monkey kidney fibroblasts, described by Gluzman, Cell, 23:175 (1981), and

other cell lines capable of expressing a compatible vector, for example, the C127, 3T3, CHO, HeLa and BHK cell lines. Mammalian expression vectors will comprise an origin of replication, a suitable promoter and enhancer, and also any necessary ribosome binding sites, polyadenylation site, splice donor and acceptor sites, transcriptional termination sequences, and 5' flanking nontranscribed sequences. DNA sequences derived from the SV40 splice, and polyadenylation sites may be used to provide the required nontranscribed genetic elements.

The polypeptide can be recovered and purified from recombinant cell cultures by methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography hydroxylapatite chromatography and lectin chromatography. Protein refolding steps can be used, as necessary, in completing configuration of the mature protein. Finally, high performance liquid chromatography (HPLC) can be employed for final purification steps.

The polypeptides of the present invention may be a naturally purified product, or a product of chemical synthetic procedures, or produced by recombinant techniques from a prokaryotic or eukaryotic host (for example, by bacterial, yeast, higher plant, insect and mammalian cells in culture). Depending upon the host employed in a recombinant production procedure, the polypeptides of the present invention may be glycosylated or may be non-glycosylated. Polypeptides of the invention may also include an initial methionine amino acid residue.

The polypeptide of the present invention is also useful for identifying other molecules which may have similar biological activity. Labeled oligonucleotides having a sequence complementary to that of the gene of the present invention are used to screen a library of human cDNA, genomic DNA or mRNA to determine which members of the library the probe hybridizes to.

The polypeptides may also be employed in accordance with the present invention by expression of such polypeptides *in vivo*, which is often referred to as "gene therapy."

Thus, for example, cells from a patient may be engineered with a polynucleotide (DNA or RNA) encoding a polypeptide *ex vivo*, with the engineered cells then being provided to a patient to be treated with the polypeptide. Such methods are well-known in the art. For example, cells may be engineered by procedures known in the art by use of a retroviral particle containing RNA encoding a polypeptide of the present invention.

Similarly, cells may be engineered *in vivo* for expression of a polypeptide *in vivo* by, for example, procedures known in the art. As known in the art, a producer cell for producing a retroviral particle containing RNA encoding the polypeptide of the present

invention may be administered to a patient for engineering cells *in vivo* and expression of the polypeptide *in vivo*. These and other methods for administering a polypeptide of the present invention by such method should be apparent to those skilled in the art from the teachings of the present invention. For example, the expression vehicle for engineering cells may be other than a retrovirus, for example, an adenovirus which may be used to engineer cells in vivo after combination with a suitable delivery vehicle.

According to the invention there is provided a method for the treatment of a patient having need to modulate P2x receptor activity, said method comprising administering a therapeutically effective amount of the polypeptide of the invention, for example by providing to the patient DNA encoding said polypeptide and expressing said polypeptide *in vivo*.

The sequences of the present invention are also valuable for chromosome identification. The sequence is specifically targeted to and can hybridize with a particular location on an individual human chromosome. Moreover, there is a current need for identifying particular sites on the chromosome. Few chromosome marking reagents based on actual sequence data (repeat polymorphisms) are presently available for marking chromosomal location. The mapping of DNAs to chromosomes according to the present invention is an important first step in correlating those sequences with genes associated with disease.

Briefly, sequences can be mapped to chromosomes by preparing PCR primers (preferably 15-25 bp) from the cDNA. Computer analysis of the cDNA is used to rapidly select primers that do not span more than one exon in the genomic DNA, thus complicating the amplification process. These primers are then used for PCR screening of somatic cell hybrids containing individual human chromosomes. Only those hybrids containing the human gene corresponding to the primer will yield an amplified fragment.

PCR mapping of somatic cell hybrids is a rapid procedure for assigning a particular DNA to a particular chromosome. Using the present invention with the same oligonucleotide primers, sublocalization can be achieved with panels of fragments from specific chromosomes or pools of large genomic clones in an analogous manner. Other mapping strategies that can similarly be used to map to its chromosome include *in situ* hybridization, prescreening with labeled flow-sorted chromosomes and preselection by hybridization to construct chromosome specific-cDNA libraries.

Fluorescence *in situ* hybridization (FISH) of a cDNA clones to a metaphase chromosomal spread can be used to provide a precise chromosomal location in one step. This technique can be used with cDNA as short as 500 or 600 bases; however, clones larger than 2,000 bp have a higher likelihood of binding to a unique chromosomal

location with sufficient signal intensity for simple detection. FISH requires use of the clones from which the EST was derived, and the longer the better. For example, 2,000 bp is good, 4,000 is better, and more than 4,000 is probably not necessary to get good results a reasonable percentage of the time. For a review of this technique, see Verma et al., Human Chromosomes: a Manual of Basic Techniques, Pergamon Press, New York (1988).

Once a sequence has been mapped to a precise chromosomal location, the physical position of the sequence on the chromosome can be correlated with genetic map data. Such data are found, for example, in V. McKusick, Mendelian Inheritance in Man (available on line through Johns Hopkins University Welch Medical Library). The relationship between genes and diseases that have been mapped to the same chromosomal region are then identified through linkage analysis (coinheritance of physically adjacent genes).

Next, it is necessary to determine the differences in the cDNA or genomic sequence between affected and unaffected individuals. If a mutation is observed in some or all of the affected individuals but not in any normal individuals, then the mutation is likely to be the causative agent of the disease.

With current resolution of physical mapping and genetic mapping techniques, a cDNA precisely localized to a chromosomal region associated with the disease could be one of between 50 and 500 potential causative genes. (This assumes 1 megabase mapping resolution and one gene per 20 kb).

Comparison of affected and unaffected individuals generally involves first looking for structural alterations in the chromosomes, such as deletions or translocations that are visible from chromosome spreads or detectable using PCR based on that cDNA sequence. Ultimately, complete sequencing of genes from several individuals is required to confirm the presence of a mutation and to distinguish mutations from polymorphisms.

The polypeptides, their fragments or other derivatives, or analogs thereof, or cells expressing them can be used as an immunogen to produce antibodies thereto. These antibodies can be, for example, polyclonal or monoclonal antibodies. The present invention also includes chimeric, single chain, and humanized antibodies, as well as Fab fragments, or the product of an Fab expression library. Various procedures known in the art may be used for the production of such antibodies and fragments.

Antibodies generated against the polypeptides corresponding to a sequence of the present invention can be obtained by direct injection of the polypeptides into an animal or by administering the polypeptides to an animal, preferably a nonhuman. The antibody so obtained will then bind the polypeptides itself. In this manner, even a sequence

encoding only a fragment of the polypeptides can be used to generate antibodies binding the whole native polypeptides. Such antibodies can then be used to isolate the polypeptide from tissue expressing that polypeptide.

For preparation of monoclonal antibodies, any technique which provides antibodies produced by continuous cell line cultures can be used. Examples include the hybridoma technique (Kohler and Milstein, 1975, Nature, 256:495-497), the trioma technique, the human B-cell hybridoma technique (Kozbor et al., 1983, Immunology Today 4:72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole, et al., 1985, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96).

Techniques described for the production of single chain antibodies (U.S. Patent 4,946,778) can be adapted to produce single chain antibodies to immunogenic polypeptide products of this invention.

This invention provides a method of screening drugs to identify those which block (antagonists) functional interaction of ligand with the human brain P2x receptor of the invention, the method comprising measuring the ability of test compound to block interaction of ligand (usually ATP or a stable analogue thereof) with the receptor. As an example, a mammalian cell or membrane preparation expressing the P2x receptor would be incubated with labeled ligand in the presence of the drug. The ability of the drug to block this interaction could then be measured. An example of such an assay combines an appropriate ligand labelled with radio-activity, eg., [^{35}S]-dATPαS, and a potential antagonist/inhibitor with membrane-bound P_{2x} receptors or recombinant forms of the P_{2x} receptor under appropriate conditions for a competitive inhibition assay, such that the number of radiolabelled molecules bound to the receptor can determine the effectiveness of the potential antagonist/inhibitor. Alternatively, the response of a known second (or third) messenger system following interaction of ligand and receptor would be measured compared in the presence or absence of the drug. Such messenger systems include but are not limited to, cAMP guanylate cyclase, ion channels or phosphoinositide hydrolysis. They may also include the measurement of extracellular pH as an index of overall cell function.

This invention also provides transgenic non-human animals comprising a polynucleotide encoding a polypeptide of the invention. Also provided are methods for use of said transgenic animals as models for mutation and SAR (structure/activity relationship) evaluation as well as in drug screens.

The present invention is also directed to antagonist/inhibitor molecules of the polypeptides of the present invention, and their use in reducing or eliminating the function of the polypeptide.

The invention further provides a method for the treatment of a patient having need to reduce or eliminate the function of the polypeptide comprising: administering to the patient a therapeutically effective amount of the antagonist/inhibitor of the invention.

An example of an antagonist is an antibody or in some cases, an oligonucleotide which binds to the polypeptide. The antagonists may be closely related proteins such that they recognize and bind to the receptor sites of the natural protein, however, they are inactive forms of the polypeptide and thereby prevent the action of ATP since receptor sites are occupied.

An example of an inhibitor is an antisense constructs prepared using antisense technology. Antisense technology can be used to control gene expression through triple-helix formation or antisense DNA or RNA, both of which methods are based on binding of a polynucleotide to DNA or RNA. For example, the 5' coding portion of the polynucleotide sequence, which encodes for the mature polypeptides of the present invention, is used to design an antisense RNA oligonucleotide of from about 10 to 40 base pairs in length. A DNA oligonucleotide is designed to be complementary to a region of the gene involved in transcription (triple helix -see Lee et al., Nucl. Acids Res., 6:3073 (1979); Cooney et al, Science, 241:456 (1988); and Dervan et al., Science, 251: 1360 (1991)), thereby preventing transcription and the production of the mature polypeptide. The antisense RNA oligonucleotide hybridizes to the mRNA *in vivo* and blocks translation of the mRNA molecule into the P_{2x} receptor (antisense - Okano, J. Neurochem., 56:560 (1991); Oligodeoxynucleotides as Antisense Inhibitors of Gene Expression, CRC Press, Boca Raton, FL (1988)). The oligonucleotides described above can also be delivered to cells such that the antisense RNA or DNA may be expressed *in vivo* to inhibit production of the P_{2x} receptor.

Another example of an antagonist/inhibitor is a small molecule which binds to and occupies the receptor binding site, thereby making the site inaccessible to its natural ligand or which binds to a related site which affects the function of ATP in an allosteric manner such that normal biological activity is prevented. Examples of small molecules include but are not limited to small peptides or peptide-like molecules.

The antagonist/inhibitors may be employed to treat conditions associated with an inappropriately high presence of ATP. These include conditions in which there is an excessive release of ATP from hyperactive neurones that normally contain ATP as a neurotransmitter. They may also include conditions in which ATP is released from

damaged cells, to affect the surrounding neurones. These actions may include the mimicry of fast synaptic events and the induction of apoptosis or of cell necrosis, based on the know ability of ATP to induce apoptosis and cell death (Zheng et al., 1991, J Cell Biol., 112, 279-288) and on the high sequence homology between at least one type of rat P2x receptor (Valera et al, 1994 above) and the apoptotic gene product RP-2, expressed by rat thymocytes during apoptosis (Owens et al., 1991, Mol Cell Biol., 11, 4177-4188).

Localisation of the P_{2X}-1 receptor and / or the P_{2X}-2 receptor within the various regions of the brain, including the frontal cortex, amygdala, caudate, cerebellum, hippocampus, substantia nigra, thalamus and spinal cord, means that therapeutic agents which modulate the activity of these receptors will be indicated for a number of important clinical conditions, including brain stroke, brain or spinal cord traumas, infection and inflammation, cognitive disorders, epilepsy, affective and mood disorders in general, including depression, various movement disorders including Parkinson's disease, Huntingtons Chorea and schizophrenia, as well as those conditions that are associated with the development of chronic or acute forms of pain.

The variable distribution of the P_{2X} receptors of the invention suggests that the clinical indication of drugs which affect a particular splice variant of the P_{2X} receptor will depend on its exact location within the brain.

The P_{2X} receptors of the invention are also present in certain tissues outside the brain, such as the heart and liver, where they will play a role in various conditions associated with tissue damage - eg., cardiac anoxia.

The polypeptides, polynucleotides and antagonist/inhibitors of the present invention may be employed in combination with a suitable pharmaceutical carrier. Such compositions, which form part of the invention, comprise a therapeutically or prophylactically effective amount of the active substance, and a pharmaceutically acceptable carrier or excipient. Such a carrier includes but is not limited to saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof. The formulation should suit the mode of administration.

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration. In addition, the active substances of the present invention may be employed in conjunction with other therapeutic compounds.

The pharmaceutical compositions may be administered in a convenient manner such as by the oral, topical, intravenous, intraperitoneal, intramuscular, subcutaneous, intranasal or intradermal routes. The active substance is administered in an amount which is effective for treating and/or prophylaxis of the specific indication. The amounts and dosage regimens of active substance and administered to a subject will depend on a number of factors such as the mode of administration, the nature of the condition being treated and the judgment of the prescribing physician. In general, the active substance will be administered in an amount of at least about x mg/kg body weight and in most cases they will be administered in an amount not in excess of about y mg/kg body weight per day, taking into account the routes of administration, symptoms, etc.

The present invention will be further described with reference to the following examples; however, it is to be understood that the present invention is not limited to such examples. All parts or amounts, unless otherwise specified, are by weight.

In order to facilitate understanding of the following examples certain frequently occurring methods and/or terms will be described.

"Plasmids" are designated by a lower case p preceded and/or followed by capital letters and/or numbers. The starting plasmids herein are either commercially available, publicly available on an unrestricted basis, or can be constructed from available plasmids in accord with published procedures. In addition, equivalent plasmids to those described are known in the art and will be apparent to the ordinarily skilled artisan.

"Digestion" of DNA refers to catalytic cleavage of the DNA with a restriction enzyme that acts only at certain sequences in the DNA. The various restriction enzymes used herein are commercially available and their reaction conditions, cofactors and other requirements were used as would be known to the ordinarily skilled artisan. For analytical purposes, typically 1 µg of plasmid or DNA fragment is used with about 2 units of enzyme in about 20 µl of buffer solution. For the purpose of isolating DNA fragments for plasmid construction, typically 5 to 50 µg of DNA are digested with 20 to 250 units of enzyme in a larger volume. Appropriate buffers and substrate amounts for particular restriction enzymes are specified by the manufacturer. Incubation times of about 1 hour at 37 C are ordinarily used, but may vary in accordance with the supplier's instructions. After digestion the reaction is electrophoresed directly on a polyacrylamide gel to isolate the desired fragment.

Size separation of the cleaved fragments is performed using 8 percent polyacrylamide gel described by Goeddel, D. *et al.*, Nucleic Acids Res., 8:4057 (1980).

"Oligonucleotides" refers to either a single stranded polydeoxynucleotide or two complementary polydeoxynucleotide strands which may be chemically synthesized.

Such synthetic oligonucleotides have no 5' phosphate and thus will not ligate to another oligonucleotide without adding a phosphate with an ATP in the presence of a kinase. A synthetic oligonucleotide will ligate to a fragment that has not been dephosphorylated.

"Ligation" refers to the process of forming phosphodiester bonds between two double stranded nucleic acid fragments (Maniatis, T., et al., Id., p. 146). Unless otherwise provided, ligation may be accomplished using known buffers and conditions with 10 units to T4 DNA ligase ("ligase") per 0.5 μg of approximately equimolar amounts of the DNA fragments to be ligated.

Unless otherwise stated, transformation was performed as described in the method of Graham, F. and Van der Eb, A., Virology, 52:456-457 (1973).

Example 1

Isolation of P_{2X} cDNA clones and DNA sequencing

Partial cDNA was obtained from a human brain frontal cortex (taken from a schizophrenia patient) cDNA library. A comparison of the sequence with the published rat P_{2X} receptors, referred to above, suggested that this EST sequence was approximately 300bp short of a full length cDNA sequence. To obtain the remaining 5' coding sequence Marathon-Ready cDNA (Clonetech Laboratories, Inc., 4030 Fabian way, Palo Alto, CA 94303-40607 USA) sythesised from adult human frontal cortex RNA was obtained and standard RACE technology used as described in methods outlined in the Marathon-Ready cDNA Kit (cat. #PT1156-1). RACE was performed using a 5' oligonucleotide (AP1 5'-CCA TCC TAA TAC GAC TCA CTA TAG GGC-3') which was supplied in Marathon-Ready kit and a 3' oligonuclotide (#1, 5'-ACA CAC AGT GGT CGC ATC TGG AAT C-3') complimentary to the 5' end of the partial cDNA sequence.

Marathon-Ready cDNA (20ng) were amplified by following standard PCR protocols and using Taq DNA polymerase (Applied Biosystems, Warrington UK) in a touch down PCR described in Don et al 1991 (Nucleic Acid Research Vol 19, 4008). The initial annealing temperature for touch down PCR started at 60°C and was reduced by 1°C every 1 cycle for 10 cycles (final annealing temperature 50°C) where it was then cycled for 20 more cycles. Each cycle also contained polymerisation (72°C, 1 min) and denaturation (94°C 1 min) steps and the PCR finished off with a final 10 min 72°C polishing step. Amplified products were then subcloned into pGEMT vectors (Promega Corp.) and transformed into DH5alpha cells (Bethesda Research Laboratories; BRL) for standard blue/white selection using standard manufacturers protocols. White colonies were selected from LBamp plates (containing Xgal and IPTG) and grown overnight for extraction of plasmid DNA using standard mini prep alkaline lysis (Molecular cloning, A laboratory manual, Maniatis *et. al* 1982 Cold Spring Harbor Laboratory, NY). DNA sequencing ulitilised the modified dideoxy nuclotide protocol decribed in Snutch et al 1991 (Neuron, Vol 7, 45-57).

First strand sequence was obtained using AP2 (5'-ACT CAC TAT AGG GCT CGA GCG GC-3'; Clontech) and SP6 primers purchased from BRL. After determination of first strand sequence a set of oligonucleotide primers #8 (5'-CCT TCC TGT TCG AGT ACG AC-3') and #9 (5'-TCC CAG ATC CGG AAT CCA AG-3') were synthesised and used to obtain the remainder of the sequence of P_{2X}-1 (SEQ ID NO 1) P_{2X}-2 (SEQ ID NO 2)

Example 2
PCR localization

Poly A^+ mRNA was purchased from Clonetech or isolated from normal human brain tissues using Trizol (methods supplied by BRL). Human genomic DNA was purchased from Promega.

To determine the expression pattern of P_{2X}-1 and P_{2X}-2 in human brain tissues, heart and liver. 25 ng of Poly A^+ mRNA from each of the human tissues was mixed with poly dT oligonucleotide primer and reverse transcribed using Superscript II following protocols supplied by BRL. The entire mix was then amplified in a 50 ul reaction mix that included 0.2uM upstream primer #8 (5'-CCT TCC TGT TCG AGT ACG AC-3') and downstream primer # 9 (5'-TCC CAG ATC CGG AAT CCA AG -3') following the PCR protocol outlined previously. The expected size of products was 226 bp and 274 bp which correspond to P_{2X}-1 and P_{2X}-2 respectively. As a control for genomic contamination identical reactions were performed using the RNA samples without reverse transcription or 100ng of genomic DNA. The reaction products were separated and visualised on a 1.2% agarose gel containing ethidium bromide. The DNA was then transferred to nylon membrane as in Snutch et al 1991 (Neuron, Vol 7, 45-57) and probed with P_{2X}-1 and P_{2X}-2 insert specific ^{32}P labelled oligonuclotide probes #7 (5'-CCC ACA CAA ACA CCC ACC CGA TGA CGT AGG CCA GGA TGA-3') and #6 (5'-GAC TCC ATT TCC ACT TCT GCC AAA TGG AGA TGG TAG CAC-3') respectively (Figure 6 & 7). The hybridization was performed with standard protocols as in Snutch et al 1991 (Neuron, Vol 7, 45-57) with a final wash temperature of 60°C in 0.5x SSC. Southern blots were then exposed directly to X-Omat R film for 72 hours. The film was developed and images were examined on an optimas imaging system.

Results: The primers used in the PCR reaction -- oligo 8 and oligo 9 -- were designed to amplify across the splice site in P_{2X}-1 and P_{2X}-2. RT-PCR with these primers on poly A+ RNA from human brain regions produced two products of 226bp and 274bp representing both P_{2X} isoforms respectively. The 226bp (Figure 6) product represents P_{2X}-1 and was amplified from total brain, amygdala, caudate, cerebellum, hippocampus, substantia nigra, thalamus, spinal cord and heart mRNAs.

According to the results shown in (Figure 7) a 274 bp amplified product representing P_{2X}-2 was produced in all areas tested;total brain, amygdala, caudate, cerebellum,

hippocampus, substantia nigra, thalamus, hypothalamus, frontal cortex, spinal cord, heart and liver.

In addition to the expected sized products, products of varying length were differentially expressed throughout the different brain regions. The extra products are ~150bp, ~340 bp, ~825bp and ~996 bp in length. Since the primer set used in the PCR reaction -- oligos 8 and oligo 9 -- are on opposite sides of a known splice junction in the P_{2x} gene, these other products represent further potential alternative splice variants of the human brain P_{2x-2} receptor.

Example 3

***In situ* localisation**

To determine the regional expression pattern of P_{2x}-1 and P_{2x}-2 in the rat brain ^{35}S labelled insert specific oligonuclotides (#7 and #6) were used for *in situ* hybridization on 10uM PFA fixed rat brain tissue sections. *In situ* hybridisation was performed using standard protocols as in Stea et al 1994 (PNAS Vol. 91, 10576-10580) with a hybridisation and final wash temperature of 42°C in 0.5 x SSC.

Tissue sections were exposed directly to X-Omat R film for 10 days. The film was developed and images were examined on an optimas imaging system. Slides were dipped in photographic emulsion (Kodak) and exposed for a further 6 weeks.

Results: Using a probe common to both P_{2x}-1 and P_{2x}-2 cDNAs show that these receptors are weakly detected in most areas of the rat brain with a stonger signal associated with the cerebellum. However, using an oligonucleotide probe specific to P_{2x}-2 strong labelling was only detected in specific rat brain regions corresponding to the striatum, frontal cortex and the dentate gyrus of the hippocampus.

Figure 1.

Human Brain P2X-1 DNA Sequence. Length 1759bp
cDNA length 1167bp coding sequence from 14bp-1180bp.
ATG is at nucleotide 14.
If the A of ATG is defined as +1

% A = 23.82	[278]
% G = 26.56	[310]
% T = 22.19	[259]
% C = 27.42	[320]

SEQ ID NO 1

GCGGGCGCCGGCCATGGCGGGCTGCTGCGCCGCGCTGGCGGCCTTCCTGTTCGAGTACGA
CACGCCGCGCATCGTGCTCATCCGCAGCCGCAAAGTGGGGCTCATGAACCGCGCCGTGCA
ACTGCTCATCCTGGCCTACGTCATCGGGTGGGTGTTTGTGTGGGAAAAGGGCTACCAGGA
AACTGACTCCGTGGTCAGCTCCGTTACGACCAAGGTCAAGGGCGTGGCTGTGACCAACAC
TTCTAAACTTGGATTCCGGATCTGGGATGTGGCGGATTATGTGATACCAGCTCAGGAGGA
AAACTCCCTCTTCGTCATGACCAACGTGATCCTCACCATGAACCAGACACAGGGCCTGTG
CCCCGAGATTCCAGATGCGACCACTGTGTGTAAATCAGATGCCAGCTGTACTGCCGGCTC
TGCCGGCACCCACAGCAACGGAGTCTCAACAGGCAGGTGCGTAGCTTTCAACGGGTCTGT
CAAGACGTGTGAGGTGGCGGCCTGGTGCCCGGTGGAGGATGACACACACGTGCCACAACC
TGCTTTTTTAAAGGCTGCAGAAAACTTCACTCTTTTGGTTAAGAACAACATCTGGTATCC
CAAATTTAATTTCAGCAAGAGGAATATCCTTCCCAACATCACCACTACTTACCTCAAGTC
GTGCATTTATGATGCTAAAACAGATCCCTTCTGCCCCATATTCCGTCTTGGCAAAATAGT
GGAGAACGCAGGACACAGTTTCCAGGACATGGCCGTGGAGGGAGGCATCATGGGCATCCA
GGTCAACTGGGACTGCAACCTGGACAGAGCCGCCTCCCTCTGCTTGCCCAGGTACTCCTT
CCGCCGCCTCGATACACGGGACGTTGAGCACAACGTATCTCCTGGCTACAATTTCAGGTT
TGCCAAGTACTACAGAGACCTGGCTGGCAACGAGCAGCGCACGCTCATCAAGGCCTATGG
CATCCGCTTCGACATCATTGTGTTTGGGAAGGCAGGGAAATTTGACATCATCCCCACTAT
GATCAACATCGGCTCTGGCCTGGCACTGCTAGGCATGGCGACCGTGCTGTGTGACATCAT
AGTCCTCTACTGCATGAAGAAAAGACTCTACTATCGGGAGAAGAAATATAAATATGTGGA
AGATTACGAGCAGGGTCTTGCTAGTGAGCTGGACCAGTGAGGCCTACCCCACACCTGGGC
TCTCCACAGCCCCATCAAAGAACAGAGAGGAGGAGGAGGGAGAAATGGCCACCACATCAC
CCCAGAGAAATTTCTGGAATCTGATTGAGTCTCCACTCCACAAGCACTCAGGGTTCCCCA
GCAGCTCCTGTGTGTTGTGTGCAGGATCTGTTTGCCCACTCGGCCCAGGAGGTCAGCAGT
CTGTTCTTGGCTGGGTCAACTCTGCTTTTCCCGCAACCTGGGGTTGTCGGGGGAGCGCTG
GCCCGACGCAGTGGCACTGCTGTGGCTTTCAGGGCTGGAGCTGGCTTTGCTCAGAAGCCT
CCTGTCTCCAGCTCTCTCCAGGACAGGCCCAGTCCTCTGAGGCACGGCGGCTCTGTTCAA
GCACTTTATGCGGCAGGGGAGGCCGCCTGGCTGCAGTCACTAGACTTGTAGCAGGCCTGG
GCTGCAGGCTTCCCCCCGACCATTCCCTGCAGCCATGCGGCAGAGCTGGCATTTCTCCTC
AGAGAAGCGCTGTGCTAAGGTGATCGAGGACCAGACATTAAAGCGTGATTTTCTTAAAAA
AAAAAAAAAAAAACTCGAG

Figure 2

Human Brain P_{2X}-2 DNA Sequence. Length 1807bp
cDNA Length=1215bp
Coding sequence from 14bp-1228bp
ATG is at nucleotide 14.
If the A of ATG is defined as +1, the 48bp insertion begins at nucleotide 135.

% A = 23.95	[291]
% G = 26.58	[323]
% T = 22.22	[270]
% C = 27.24	[331]

SEQ ID NO 2

GCGGGCGCCGGCCATGGCGGGCTGCTGCGCCGCGCTGGCGGCCTTCCTGTTCGAGTACGA
CACGCCGCGCATCGTGCTCATCCGCAGCCGCAAAGTGGGGCTCATGAACCGCGCCGTGCA
ACTGCTCATCCTGGCCTACGTCATCGGGTGCTACCATCTCCATTTGGCAGAAGTGGAAAT
GGAGTCCCCTAGAAGGTGGGTGTTTGTGTGGGAAAAGGGCTACCAGGAAACTGACTCCGT
GGTCAGCTCCGTTACGACCAAGGTCAAGGGCGTGGCTGTGACCAACACTTCTAAACTTGG
ATTCCGGATCTGGGATGTGGCGGATTATGTGATACCAGCTCAGGAGGAAAACTCCCTCTT
CGTCATGACCAACGTGATCCTCACCATGAACCAGACACAGGGCCTGTGCCCCGAGATTCC
AGATGCGACCACTGTGTGTAAATCAGATGCCAGCTGTACTGCCGGCTCTGCCGGCACCCA
CAGCAACGGAGTCTCAACAGGCAGGTGCGTAGCTTTCAACGGGTCTGTCAAGACGTGTGA
GGTGGCGGCCTGGTGCCCGGTGGAGGATGACACACACGTGCCACAACCTGCTTTTTTAAA
GGCTGCAGAAAACTTCACTCTTTTGGTTAAGAACAACATCTGGTATCCCAAATTTAATTT
CAGCAAGAGGAATATCCTTCCCAACATCACCACTACTTACCTCAAGTCGTGCATTTATGA
TGCTAAAACAGATCCCTTCTGCCCCATATTCCGTCTTGGCAAAATAGTGGAGAACGCAGG
ACACAGTTTCCAGGACATGGCCGTGGAGGGAGGCATCATGGGCATCCAGGTCAACTGGGA
CTGCAACCTGGACAGAGCCGCCTCCCTCTGCTTGCCCAGGTACTCCTTCCGCCGCCTCGA
TACACGGGACGTTGAGCACAACGTATCTCCTGGCTACAATTTCAGGTTTGCCAAGTACTA
CAGAGACCTGGCTGGCAACGAGCAGCGCACGCTCATCAAGGCCTATGGCATCCGCTTCGA
CATCATTGTGTTTGGGAAGGCAGGGAAATTTGACATCATCCCCACTATGATCAACATCGG
CTCTGGCCTGGCACTGCTAGGCATGGCGACCGTGCTGTGTGACATCATAGTCCTCTACTG
CATGAAGAAAAGACTCTACTATCGGGAGAAGAAATATAAATATGTGGAAGATTACGAGCA
GGGTCTTGCTAGTGAGCTGGACCAGTGAGGCCTACCCCACACCTGGGCTCTCCACAGCCC
CATCAAAGAACAGAGAGGAGGAGGAGGGAGAAATGGCCACCACATCACCCCAGAGAAATT
TCTGGAATCTGATTGAGTCTCCACTCCACAAGCACTCAGGGTTCCCCAGCAGCTCCTGTG
TGTTGTGTGCAGGATCTGTTTGCCCACTCGGCCCAGGAGGTCAGCAGTCTGTTCTTGGCT
GGGTCAACTCTGCTTTTCCCGCAACCTGGGGTTGTCGGGGGAGCGCTGGCCCGACGCAGT
GGCACTGCTGTGGCTTTCAGGGCTGGAGCTGGCTTTGCTCAGAAGCCTCCTGTCTCCAGC
TCTCTCCAGGACAGGCCCAGTCCTCTGAGGCACGGCGGCTCTGTTCAAGCACTTTATGCG
GCAGGGGAGGCCGCCTGGCTGCAGTCACTAGACTTGTAGCAGGCCTGGGCTGCAGGCTTC
CCCCCGACCATTCCCTGCAGCCATGCGGCAGAGCTGGCATTTCTCCTCAGAGAAGCGCTG
TGCTAAGGTGATCGAGGACCAGACATTAAAGCGTGATTTTCTTAAAAAAAAAAAAAAAAA
ACTCGAG

Figure 3.
Human Brain P2X-1 Predicted Amino Acid Sequence.

Translated DNA Sequence hBrain P2X(14bp-1180bp)
With Standard Genetic Code

Molecular Weight 43.3737 kDa
388 Amino Acids

SEQ ID NO3
MAGCCAALAAFLFEYDTPRIVLIRSRKVGLMNRAVQLLILAYVIGWVFVWEKGY
QETDSVVSSVTTKVKGVAVTNTSKLGFRIWDVADYVIPAQEENSLFVMTNVILT
MNQTQGLCPEIPDATTVCKSDASCTAGSAGTHSNGVSTGRCVAFNGSVKTCEVA
AWCPVEDDTHVPQPAFLKAAENFTLLVKNNIWYPKFNFSKRNILPNITTTYLKSCI
YDAKTDPFCPIFRLGKIVENAGHSFQDMAVEGGIMGIQVNWDCNLDRAASLCLPR
YSFRRLDTRDVEHNVSPGYNFRFAKYYRDLAGNEQRTLIKAYGIRFDIIVFGKAG
KFDIIPTMINIGSGLALLGMATVLCDIIVLYCMKKRLYYREKKYKYVEDYEQGLA
SELDQ.

Figure 4
Human Brain P_{2X}-2 Predicted Amino Acid Sequence.

Translated DNA Sequence hBrain P2X-1(14bp-1228bp)
With Standard Genetic Code

Molecular Weight 45326.20 Daltons
404 Amino Acids

SEQ ID NO 4
MAGCCAALAAFLFEYDTPRIVLIRSRKVGLMNRAVQLLILAYVIGCYHLHLAEVE
MESPRRWVFVWEKGYQETDSVVSSVTTKVKGVAVTNTSKLGFRIWDVADYVIP
AQEENSLFVMTNVILTMNQTQGLCPEIPDATTVCKSDASCTAGSAGTHSNGVSTG
RCVAFNGSVKTCEVAAWCPVEDDTHVPQPAFLKAAENFTLLVKNNIWYPKFNFS
KRNILPNITTTYLKSCIYDAKTDPFCPIFRLGKIVENAGHSFQDMAVEGGIMGIQV
NWDCNLDRAASLCLPRYSFRRLDTRDVEHNVSPGYNFRFAKYYRDLAGNEQRTL
IKAYGIRFDIIVFGKAGKFDIIPTMINIGSGLALLGMATVLCDIIVLYCMKKRLYYR
EKKYKYVEDYEQGLASELDQ.

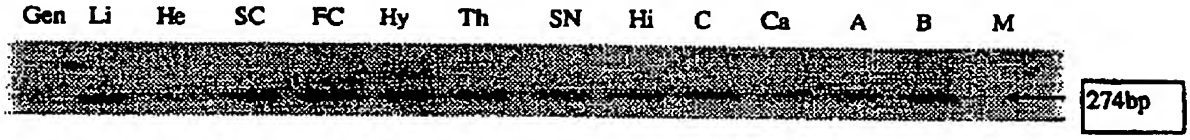
Figure 5
Human Brain P_{2X}-3 Predicted Amino Acid Partial Sequence.

SEQ ID NO 5
MAGCCAALAAFLFEYDTPRIVLIRSRKVGLMNRAVQLLILAYVIGPAFLKAAENFT
LLVKNNIWYPKFNFSKRNILPNITTTYLKSCIYDAKTDPFCPIFRLGQDSGRT

Figure 6

PCR Localisation

Southern hybridisation of RT-PCR product produced by oligo nucleotide primers 8 & 9 on human brain poly A+ RNA and control tissues. Blot was hybridised with ^{32}P labelled oligonucleotide primer 6 (hP_{2X}-2 specific).

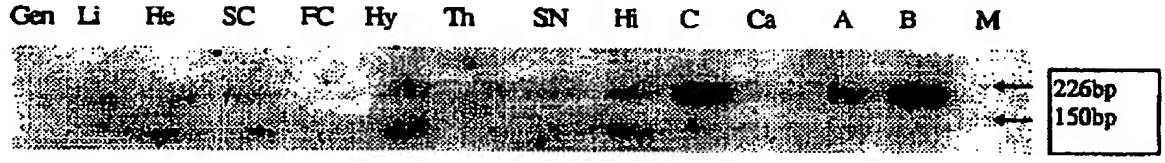


Gen: Genomic DNA
Li: Liver
He: Heart
SC: Spinal cord
FC: Frontal cortex
Hy: Hypothalamus
Th: Thalamus
SN: Substantia Nigra
Hi: Hippocampus
C: Cerebellum
Ca: Caudate
A: Amygdala
B: Whole Brain

Figure 7

PCR Localisation

Southern hybridisation of RT-PCR product produced by oligo nucleotide primers 8 & 9 on human brain poly A+ RNA and control tissues. Blot was hybridised with ^{32}P labelled oligonucleotide primer 7 (hP_{2X}-1 specific).



Gen: Genomic DNA
Li: Liver
He: Heart
SC: Spinal cord
FC: Frontal cortex
Hy: Hypothalamus
Th: Thalamus
SN: Substantia Nigra
Hi: Hippocampus
C: Cerebellum
Ca: Caudate
A: Amygdala
B: Whole Brain

Claims

1. A polynucleotide encoding a polypeptide which is a human brain P_{2X} receptor polypeptide:

(a) having the deduced amino acid sequence of SEQ ID NO 3 or 4;

(b) characterised by the deduced amino acid sequence of SEQ ID NO 5;

(c) characterised by the amino acid sequence comprising residues 1 to 45 of SEQ ID NO 3; or

(d) or characterised by the amino acid sequence comprising residues 240-388 of SEQ ID NO 3;

or a fragment, analogue or derivative thereof.

2. The polynucleotide of claim 1 wherein the polynucleotide is DNA.

3. The polynucleotide of claim 1 wherein the polynucleotide is RNA.

4. The polynucleotide of claim 2 wherein the polynucleotide is genomic DNA.

5. The polynucleotide of any preceding claim wherein said polynucleotide encodes the polypeptide of SEQ ID NO 3 or 4 or characterised by the deduced amino acid sequence of SEQ ID NO 5.

6. The polynucleotide of claim 2 having the DNA sequence given in SEQ ID NO 1 or 2.

7. The polynucleotide of any preceding claim in isolated form.

8. A vector containing the DNA of any one of claims 2, 4, 5, 6, or 7.

9. A host cell genetically engineered with the vector of claim 8.

10. A process for producing a polypeptide comprising: expressing from the host cell of claim 9 the polypeptide encoded by said DNA.

11. A process for producing cells capable of expressing a polypeptide comprising genetically engineering cells with the vector of claim 8.

12. A polynucleotide hybridizable to the polynucleotide of any one of claims 1 to 7 and encoding a polypeptide having substantially the same biological function or activity as the polypeptide of SEQ ID NO 3 or 4 or characterised by the deduced amino acid sequence of SEQ ID NO 5.

13. A human brain P_{2X} receptor polypeptide:

(a) having the deduced amino acid sequence of SEQ ID NO 3 or 4;

(b) characterised by the deduced amino acid sequence of SEQ ID NO 5;

(c) characterised by the amino acid sequence comprising residues 1 to 45 of SEQ ID NO 3; or

(d) or characterised by the amino acid sequence comprising residues 240-388 of SEQ ID NO 3;

or a fragment, analogue or derivative thereof.

14. The polypeptide of Claim 13 wherein the polypeptide has the amino acid sequence of SEQ ID NO 3 or 4 or characterised by the deduced amino acid sequence of SEQ ID NO 5.

15. The polypeptide of claim 13 or 14 in isolated form.

16. A method of screening drugs to identify those which block functional interaction of ligand with the human brain P2x receptor of claim 13 or 14, the method comprising measuring the ability of test compound to block interaction of ligand with the receptor.

17. A compound identified by the method of claim 16.

18. An antagonist/inhibitor of the polypeptide of claim 13 or 14.

19. A pharmaceutical composition comprising the polynucleotide of claim 1 or 12, a polypeptide of claim 13, a compound of claim 17 or an antagonist of claim 18 and a pharmaceutically acceptable carrier.

20. A method for the treatment of a patient having need to reduce or eliminate the function of the polypeptide of claim 13 or 14 comprising: administering to the patient a therapeutically effective amount of the compound of claim 17 or antagonist/inhibitor of the claim 17.

21. The use of a compound of claim 16 or antagonist/inhibitor of claim 17 for the manufacture of a medicament for use in therapy.

INTERNATIONAL SEARCH REPORT

Int. ..onal Application No
PCT/GB 96/01034

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C07K14/705 C07K16/28 C12N15/62 C12Q1/68
G01N33/566 G01N33/50 A61K38/17 //A61K48/00,A01K67/027,
C12N15/11

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 33048 A (GLAXO GROUP LTD ;VALERA SOLEDAD (CH); BUELL GARY NUTTER (CH)) 7 December 1995 cited in the application see page 49 - page 53	12
A	see page 13, line 21-29 see page 29, line 1 - page 30, line 10 see page 33, line 9 - page 35, line 29 see page 37, line 16 - page 39, line 21 see claims 16-18 see figures 12-15	16-21

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

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Date of the actual completion of the international search	Date of mailing of the international search report
30 January 1997	12. 02. 97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Macchia, G

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inte. onal Application No
PCT/GB 96/01034

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	THE JOURNAL OF NEUROSCIENCE, vol. 16, no. 2, 15 January 1996, pages 448-455, XP000616491 SEGUELA ET AL.: "A novel neuronal P2x ATP receptor ion channel with widespread distribution in the brain" see page 449; figure 1	12
A	BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 220, no. 1, 7 March 1996, pages 196-202, XP002023836 WANG ET AL.: "Cloning and pharmacological characterization of a fourth P2x receptor subtype widely expressed in brain and peripheral tissues including various endocrine tissues" see page 198; figure 1	12

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB 96/01034

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 20
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although this claim is directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte. onal Application No
PCT/GB 96/01034

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9533048	07-12-95	AU-A- 2566595	21-12-95